PSCA and MUC1 in Non-small-cell Lung Cancer as Targets of Chimeric Antigen Receptor T cells

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Abstract

In recent years, immunotherapies, such as those involving chimeric antigen receptor (CAR) T cells, have become increasingly promising approaches to non-small-cell lung cancer (NSCLC) treatment. In this study, we explored the anti-tumor potential of prostate stem cell antigen (PSCA)-redirected CAR T and mucin 1 (MUC1)-redirected CAR T cells in tumor models of NSCLC. First, we generated patient-derived xenograft (PDX) mouse models of human NSCLC that maintained the antigenic profiles of primary tumors. Next, we demonstrated the expression of PSCA and MUC1 in NSCLC, followed by the generation and confirmation of the specificity and efficacy of PSCA- and MUC1-targeting CAR T cells against NSCLC cell lines in vitro. Finally, we demonstrated that PSCA-targeting CAR T cells could efficiently suppress NSCLC tumor growth in PDX mice and synergistically eliminate $PSCA^{+}MUC1^{+}$ tumors when combined with MUC1-targeting CAR T cells. Taken together, our studies demonstrate that PSCA and MUC1 are both promising CAR T cell targets in NSCLC and that the combinatorial targeting of these antigens could further enhance the anti-tumor efficacy of CAR T cells.

Key words: Non-small-cell lung cancer, Patient-derived xenograft, CAR T, PSCA, MUC1

Introduction

Globally, lung cancer is the greatest killer among all cancers[1,2], and non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all cases of lung cancer[3,4]. Current therapeutic strategies, including surgery, radiation and chemotherapy, have not yielded significant survival benefits. Tyrosine kinase inhibitors (TKIs) targeting EGFR and ALK have been widely used to treat NSCLC, but frequent resistance to these drugs develops due to acquired mutations of EGFR[5-7] and of ALK[8]. Furthermore, recently introduced CTLA4, PD-1 and PD-L1 immune checkpoint inhibitors have had no[9] or only moderate effects on NSCLC[10-13]. Therefore, novel treatment regimens are still needed.

Chimeric antigen receptor (CAR) T cells that target CD19 have generated exciting results in leukemia and lymphoma[14-16]. However, the broad applicability of these cells for solid cancer is limited by the paucity of truly tumor-specific target antigens. Additionally, the heterogeneity of tumor-associated antigens (TAAs) in solid cancers complicates CAR T cell therapies, as the targets may differ among various cancers and even patients of a same cancer. Therefore, it is important to define the TAA profile of a solid cancer before using TAA-oriented personalized CAR T cell immunotherapies.

Few CAR T cell antigens have been targeted to treat NSCLC. Glypican-3 was recently reported as a promising target for lung squamous cell carcinoma[17]. In a phase I clinical trial of anti-epidermal growth factor receptor (EGFR) CAR T cells for lung cancer, only 2 of 11 patients achieved partial responses[18]. The expression of MUC1, a

transmembrane glycoprotein, is aberrantly upregulated in many types of cancer, including NSCLC[19]. A trial of MUC1-targeting CAR T cells is currently recruiting patients with four types of solid cancers, including NSCLC (ClinicalTrials.gov Identifier: NCT02587689). Hence, MUC1 is a promising CAR T cell target in NSCLC.

Prostate stem cell antigen (PSCA) is a glycosylphosphoinositol-anchored cell surface antigen[20] that is overexpressed mainly in prostate cancer[21], although its expression has also been reported in other tumors such as gallbladder adenocarcinoma[22] and gastric cancer[23]. Surprisingly, PSCA is frequently overexpressed in NSCLC[20], although this requires confirmation. Antibody-based, PSCA-targeted therapies, as well as a peptide vaccine, have been explored for the treatment of prostate cancer[24-28]. Furthermore, PSCA-targeting CAR T cells have been used to treat pancreatic cancer in humanized mice[29], and clinical trials of anti-PSCA CAR T cells for the treatment of prostate, bladder and pancreatic cancers are ongoing (ClinicalTrials.gov Identifier, NCT02092948 and NCT02744287). It remains unknown, however, whether anti-PSCA CAR T cells could be used to treat NSCLC.

Patient-derived xenograft (PDX) models have been widely used in translational cancer research[30], which faithfully resemble the original tumors from which they were developed and this similarity is maintained across passages[31]. In this study, we generated a PDX model of NSCLC in which we detected strong histological expression of PSCA and weak expression of MUC1. We subsequently proved the capacities of PSCA- and MUC1-targeted CAR T cells to recognize and kill NSCLC

cells expressing the respective target antigens. Finally, we observed enhanced efficacy of a combination of both PSCA- and MUC1-targeted CAR T cells against double-positive NSCLC samples. Our results suggest that PSCA and MUC1 are NSCLC-specific targets of CAR T cells and indicate that combinatorial antigen targeting could enhance the anti-tumor efficacy of these cells.

Results

1. PDX models retained molecular phenotype of NSCLC cells

Using TALEN-mediated gene targeting, we previously generated a NOD-SCID-IL2Rγ$^{-/-}$ strain (NSI) of mice capable of engrafting and modeling human hematopoietic cells[32]. Here, we generated human NSCLC PDX mice by subcutaneously or intravenously implanting dissected primary tumor masses or cell suspensions that could be serially transplanted and engrafted in NSI mice. Immunohistochemistry results showed that HLA^{+}NSCLC cells from a patient (patient P2) that had been engrafted in the lungs of NSI mice expressed E-cadherin, but not vimentin, during both the first and second passages after transplantation (Fig. 1A). Moreover, NSCLC cells from third passage of another patient (patient P1) metastasized from the initial subcutaneous implants to the livers and spleens of NSI mice, and tumor cells from all locations unanimously expressed vimentin but not E-cadherin (Fig. 1B). Therefore, PDX models of NSCLC retained the molecular phenotypes of NSCLC cells across different passages, as well as in different organs of metastasis.

2. Frequent expression of PSCA in NSCLC

NSCLC samples from 8 patients were successfully engrafted in NSI mice to generate PDX models (Table 1). We subsequently harvested the tumors and evaluated the expression of PSCA and MUC1. Although the latter is frequently overexpressed in NSCLC[19], only 2 of the 8 patients in our study expressed this antigen; in contrast, 7 patients, including 2 patients who expressed MUC1, expressed PSCA (Fig. 2). Collectively, our results demonstrate the frequent expression of PSCA in human NSCLC cells, consistent with a previous report[20]. The co-expression of PSCA and MUC1 in patients with NSCLC prompted an attempt to evaluate the efficacy of combination CAR T cells for dual antigen targeting in our PDX models.

3. Generation of CAR T cells targeting PSCA and MUC1

To redirect T lymphocytes to PSCA and MUC1, we used a second-generation PSCA-specific CAR and MUC1-specific CAR, which respectively consisted of the single-chain variable fragments (scFvs) derived from the humanized 1G8 anti-PSCA antibody[33] and anti-MUC1 HMFG2 monoclonal antibody[34], and signaling domains from the costimulatory molecule CD28 and the CD3 ζ (Fig. 3A). Lentiviral vectors encoding green fluorescent protein (GFP; negative control), CAR-PSCA and CAR-MUC1 were transfected into pre-activated human T cells to generate GFP T, CAR-PSCA T and CAR-MUC1 T cells, respectively. Transduction efficiencies were measured as the percentages of GFP^+ cells (Fig. 3B). We used reverse transcription

polymerase chain reaction (PCR) analyses of the scFv sequences to further confirm the expression of anti-PSCA CAR and anti-MUC1 CAR in T cells (Fig. 3C).

4. CAR-PSCA T cells and CAR-MUC1 T cells specifically targeted PSCA$^+$ and MUC1$^+$ lung cancer cells, respectively, in vitro

We evaluated the specificity and efficacy of CAR-PSCA T cells against lung cancer cell lines in vitro. First, in a PSCA expression analysis of three lung cancer cell lines, A549, H23 and H460, only A549 cells were found to strongly express PSCA (Fig. 4A). Immunohistochemistry analyses also consistently detected PSCA in A549 cells (Fig. 2). A luciferase-based in vitro killing assay demonstrated that CAR-PSCA T cells specifically killed A549GL and H23-PSCA-GL cells (Fig. 4B). Enzyme-linked immunosorbent assay (ELISA) results showed the PSCA-specific induction of IL-2 and IFN-γ production in supernatants from the killing assay (Fig. 4C). Taken together, these findings indicate that CAR-PSCA T cells can recognize and kill PSCA$^+$ cells in vitro. We also confirmed the specificity and efficacy of CAR-MUC1 T cells. Like PSCA, MUC1 was only detected on A549 cells, but not H23 and H460 cells (Fig. 4D). CAR-MUC1 T cells killed A549GL and H23-MUC1-GL cells, but not H460GL or H23GL cells, in vitro (Fig. 4E). We additionally observed MUC1-specific induction of IL-2 and IFN-γ production in culture supernatants (Fig. 4F). Next, A549, H460, H23 and H23-PSCA cells were transduced with a lentiviral vector expressing GFP and luciferase (Fig. 4G), and H23-PSCA-GL and H23-MUC1-GL cells were

generating by transducing lentiviral vectors encoding PSCA and MUC1 into H23GL cells (Fig. 4H).

5. CAR-PSCA T cells were efficacious against PSCA$^+$NSCLC in PDX mice

We used a PDX model generated from the PSCA$^+$, MUC1$^-$ tumor of one patient (patient P2) to further confirm the efficacy of CAR-PSCA T cells against NSCLC (Fig. 5A). Briefly, dissected tumor masses (~2 mm × 2 mm) were subcutaneously transplanted in NSI mice to generate PDX mice, which subsequently received two infusions of T cells (Fig. 5B); the tumors were calibrated until day 40. NSCLC tumor mass growth was significantly suppressed by CAR-PSCA T cells, but not by CAR-MUC1 T cells (Fig. 5C). On day 40, the smallest tumors were those in mice treated with CAR-PSCA T cells (Fig. 5D), and tumors treated with CAR-PSCA T cells had much lower weights than those left untreated or treated with GFP T cells (Fig. 5E); however, no significant difference was found when CAR-MUC1 T cells were used, further suggesting that our CAR T cells recognized and killed NSCLC PDX tumors in an antigen-dependent manner. These results prove the efficacy of CAR-PSCA T cells against PSCA$^+$ NSCLC in PDX mice.

6. CAR-PSCA T and CAR-MUC1 T cells synergistically inhibited NSCLC growth in PDX mice

We next evaluated the efficacy of a combination of CAR-PSCA T and CAR-MUC1 T cells in a NSCLC PDX model generated from another patient (patient

P8) whose tumor expressed both PSCA and MUC1 (Fig. 5A). PDX mice were untreated (blank) or treated with identical numbers of GFP T, CAR-PSCA T, CAR-MUC1 T, or a 1:1 mix of CAR-PSCA T CAR-MUC1 T cells (Fig. 5F). Tumor growth was dramatically inhibited by CAR-PSCA T cells, CAR-MUC1 T cells and combined T cells (Fig. 5G-H). Furthermore, the tumor weights in mice treated with combined CAR T cells were significantly less than the weights in mice treated with a single type of CAR T cells (Fig. 5J–K). Collectively, the combination of CAR-PSCA and CAR-MUC1 T cells exhibited superior efficacy against NSCLC, compared with each cell type alone.

Discussion

The treatment of a majority of patients with solid cancers would require the development of genetically redirected T cells that target private somatic mutations, or neoantigens[35]. However, this is an arduous task, given the heterogeneity of the mutational landscape within a tumor mass and between metastases. Despite the paucity of tumor-specific antigens shared across various types of solid cancers and among patients within one type of cancer, the TAAs of a single specific cancer are much less heterogeneous than neoantigens. Therefore, CAR T cell immunotherapy remains important during the development of neoantigen targeting techniques.

Although numerous TAAs have been detected in NSCLC[36], few have been targeted by CAR T cells[37,38]. The use of these few non-cancer-specific antigens, which include FAP[39], EGFR[40], mesothelin[41] and glypican-3[17], has led to poor or undefined

therapeutic outcomes in patients. It is therefore important to broaden the NSCLC-specific targets of CAR T cells. PSCA-targeting CAR T cells have been developed[29,42,43] and are ready for use in clinical trials of safety in patients with prostate, bladder and pancreatic cancers (ClinicalTrials.gov Identifier: NCT02092948 and NCT02744287). Additionally, a trial of MUC1-targeting CAR T cells is currently recruiting patients for NSCLC (ClinicalTrials.gov Identifier: NCT02587689). In this study, we frequently detected PSCA and MUC1 expression in NSCLC cells and thereby demonstrated the usefulness of anti-PSCA and anti-MUC1 CAR T cells.

Dual targeting of erbB2 and MUC1 by T cells expressing both CARs has been reported to deliver complimentary signals, enhance CAR T cell proliferation, but reduce IL-2 production[44]. Combinatorial antigen recognition of PSCA and PSMA by a CAR that provides suboptimal activation and a chimeric costimulatory receptor (CCR) respectively has been reported to improve specificity and reduce off-target effects[42]. This strategy requires two types of chimeric receptors expressing on T cells together. In this study, we tested dual targeting of PSCA and MUC1 by mixed CAR T cells targeting either PSCA or MUC1 in NSCLC PDX models. Cancerous cells within a tumor mass may not unanimously express a single specific antigen, and even the $PSCA^{+}MUC1^{+}$ samples in our study might contain single positive cancerous cells, therefore, a CAR T cell-based combinatorial targeting strategy may broaden the populations of targeted cancerous cells.

Although the CAR-PSCA and CAR-MUC1 T cells used in our study caused considerable reductions in tumor sizes, they could not completely eradicate NSCLC in

PDX mice. This result could be attributed to the poor survival of CAR T cells in the immunosuppressive PDX tumor microenvironment. Strategies to improve the survival and infiltrating capacities of CAR T cells, such as optimized co-stimulation[45-47] and cytokine co-expression[47-51], are worthy of exploration for all CAR T cells in solid tumors.

Overall, we have demonstrated that because PSCA and MUC1 are both rational targets in NSCLC, PSCA- and MUC1-targeting CAR T cells might comprise novel therapeutic agents for patients with NSCLC. Our results also suggest that a mixture of CAR T cells with different specificities could target simultaneously expressed tumor antigens and lead to better therapeutic outcomes.

Materials and Methods

Vector Design

To generate CAR-PSCA and CAR-MUC1 lentiviral vectors, scFvs derived from the humanized 1G8 anti-PSCA antibody[33] and anti-MUC1 HMFG2 monoclonal antibody[34] were codon optimized and synthesized by Genscript (Piscataway, NJ, USA). These scFvs were cloned into a CAR-encoding vector backbone comprising the CD8a leader sequence, human IgD hinge, portions of the CD28 transmembrane domains and the CD28 and CD3 ζ endodomains within the second-generation lentiviral vector pWPXLd. The amino acid (aa) sequences of the CARs were CD8a leader (aa 1-21), scFv, IgD hinge (aa 187-289), CD28 (aa 153-220), CD3 ζ (aa 52-163).

Lentivirus production and transduction of primary human T cells

Lentivirus particles were produced in HEK-293T cells via polyethyleneimine (Sigma-Aldrich, St Louis, MO, USA) transfection. The pWPXLd-based lentiviral plasmid and two packaging plasmids, psPAX2 and pMD.2G, were co-transduced into HEK-293T cells. Lentivirus-containing supernatants were harvested at 48 and 72 hours post-transduction and filtered through a 0.45-um filter. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats of healthy donors using Lymphoprep (Fresenius Kabi Norge, AS, Berg i Østfold, Norway). T cells were negatively selected from PBMCs using a MACS Pan T Cell Isolation Kit (Miltenyi Biotec, Bergish Gladbach, Germany) and activated using microbeads coated with anti-human CD3, anti-human CD2 and anti-human CD28 antibodies (Miltenyi Biotec) at a 3:1 bead:cell ratio for 3 days in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 2 mM glutamine and 1% penicillin/streptomycin. On day 3 post-activation, T cells were transfected with CAR lentiviral supernatants in the presence of 8 μg /ml polybrene (Sigma). Twelve hours post-transfection, T cells were cultured in fresh media containing IL-2 (300 U/mL); subsequently, fresh media was added every 2–3 days to maintain cell density within the range of 0.5–1 × 10^6/mL. Healthy PBMC donors and all patients who provided primary patient specimens gave informed consent to the use of their samples for research purposes, and all procedures were approved by the Research Ethics Board of the Guangzhou Institutes of Biomedicine and Health (GIBH).

Cells and culture conditions

HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA). A549 (human lung adenocarcinoma), H23 (human lung adenocarcinoma), and H460 (human large cell lung cancer) cell lines were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI-1640. Luciferase-GFP expressing cell lines (A549GL, H23GL and H460GL) were generated through transfection of the parental cell line with a lentiviral supernatant containing luciferase-2A-GFP and were sorted for GFP expression on a FACS Aria™ cell sorter (BD Biosciences, San Jose, CA, USA). H23-PSCA-GL or H23-MUC1-GL cells were generated by transfecting H23 cells with lentiviral supernatant. DMEM and RPMI-1640 media were supplemented with 10% heat-inactivated FBS (Gibco/Life Technologies), 10 mM HEPES, 2 mM glutamine (Gibco/Life Technologies) and 1% penicillin/streptomycin. All cells were cultured at 37℃ in an atmosphere of 5% carbon dioxide.

Flow cytometry

All samples were analyzed using an LSR Fortessa or C6 flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA). The following antibodies were used: PSCA (clone 7F5) from Santa Cruz Biotechnology (Dallas, TX, USA) and anti-human CD227 (MUC-1, clone 16A[52]) from Biolegend (San Diego, CA, USA). The clone 16A binds to glycopeptide RPAPGS(GalNAc)TAPPAHG of MUC1 (MUC1-Tn) with high affinity[52]. The scFv of HMFG2 (used to engineer the CAR) can recognize a range of tumor-associated MUC1 glycoforms, such as Tn, STn, T and ST (binds to MUC1-Tn and MUC1- STn

with higher affinity). HMFG2 has the broadest capacity for strong binding to tumor-associated MUC1 glycoforms[34]. So the CAR-MUC1 T cells we generated can recognize the 16A positively stained cells.

In vitro tumor killing assays and cytokine release assays

A549GL, H23GL, H23-PSCA-GL or H23-MUC1-GL target cells were incubated with GFP T, CAR-PSCA T or CAR-MUC1 T cells at the indicated ratios in triplicate wells of U-bottomed 96-well plates. Supernatants were collected from wells with E:T ratios of 1:1 and subjected to an analysis of IL-2 and IFN-γ concentrations using ELISA kits (eBioscience, San Diego, CA, USA). Cells were treated with 100 μl/well of the luciferase substrate D-luciferin (potassium salt, 150 μg/ml; Cayman Chemical, Ann Arbor, MI, USA), and target cell viability was monitored using a microplate reader at a 450-nm excitation wavelength. Background luminescence was negligible (<1% of the signal from wells containing only target cells); therefore, the viability percentage (%) was equal to the experimental signal/maximal signal×100, and the killing percentage was equal to 100 - viability percentage.

PDX models for CAR T cell treatment

We used 6–8-week-old NSI mice according to protocols approved by the Institutional Animal Care and Use Committee of GIBH. All mice were maintained in specific pathogen-free (SPF)-grade cages and provided with autoclaved food and water. Surgical tumor samples were obtained from the Sun Yat-Sen University Cancer Center (Guangzhou, China) with informed consent of the patients; tumors were cut into 2 mm × 2 mm pieces and directly transplanted subcutaneously without matrigel

or other additives into 3–6 immunodeficient NSI mice within a 30-min period. Tumors that reached an approximate size <1000 mm^3 were removed and passaged to other NSI mice. On days 7 and 10, according to the doses used in other reports[53,54], 5×10^6 total T cells were injected through the tail vein into each NSCLC-burdened NSI mouse. Tumors were measured every 4 days with a caliper to determine the subcutaneous growth rate. The tumor volume was calculated using the following equation: (length × $width^2$)/2.

Reverse Transcription (RT-PCR)

mRNA was extracted from cells using RNeasy mini kit (Qiagen, Stockach, Germany) and reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (Takara, Shiga, Japan). The following primers were used:

β-ACTIN forward: 5' AGAGCTACGAGCTGCCTGAC 3'

β-ACTIN reverse: 5' AGCACTGTGTTGGCGTACAG 3'

scFv of CAR-PSCA forward: 5' CTCTGTGGGGGATAGGGTCA 3'

scFv of CAR-PSCA reverse: 5' TCACAAGATTTGGGCTCGCT 3'

scFv of CAR-MUC1 forward: 5' TCGGTGGAGGAACCAAACTG 3'

scFv of CAR-MUC1 reverse: 5' CCTCCCTTTCACAGACTCCG 3'

Immunohistochemistry

Tumor tissue sections were fixed with 10% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 4 μm, and stained using a standard hematoxylin and eosin technique. Paraffin sections were also immunostained with antibodies specific for E-cadherin (ZA0565), vimentin (ZA-0511), PSCA (ZA-0158), MUC1 (ZM-0391) and

HLA (Abcam, Cambridge, UK) overnight at 4 ℃, followed by secondary staining with secondary goat anti-mouse or goat anti-rabbit Ig (PV-9000) (ZSGB-BIO, Beijing, China). Images of all sections were obtained with a microscope (DMI6000B; Leica Microsystems, Wetzlar, Germany).

Statistics

Data are presented as means ± standard errors of the means. Student's t-test was used to determine the statistical significance of differences between samples, and a P value <0.05 was considered to indicate a significant difference. All statistical analyses were performed using Prism software, version 5.0 (GraphPad, Inc., San Diego, CA, USA).

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Conflict of Interest Statement

The authors declare no competing financial interests.

Authors and Contributors

XW, YL, and JL contributed to the conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing. LQ, YX, RZ, and QL contributed to the provision of study material or patients, collection and/or assembly of data. BL, SL, SW, and QW provided animal care and administrative supports. YY and DP contributed to the conception and design and provided financial support. YY, MP, FY, YL, XZ, YW, and PL contributed to the conception and design. XW and PL contributed to the conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript and provided financial support. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Generation and molecular characterization of patient-derived xenograft (PDX) models of non-small-cell lung cancer. (A) Hematoxylin and eosin (H&E) staining and immunohistochemistry detection of human leukocyte antigen (HLA), E-cadherin and vimentin in tumor sections from both the first and second passages of PDX mice for patient P2 (see from figure 2). Although HLA^+ cells from PDX mice also expressed E-cadherin, cells from both passages were negative for vimentin. (B) H&E staining and immunohistochemistry detection of HLA, E-cadherin and vimentin in sections from liver, spleen and subcutaneous (s.c.) tissue. All sections were from a single mouse among the third passage of PDX mice for patient P1 (see from figure 2). Scale bar = 20 μ m.

Figure 2. Sections of tumors from eight patient-derived xenograft (PDX) models. Representative images correspond to tumors derived from eight patients; all sections were stained with hematoxylin and eosin (H&E) and antibodies against human leukocyte antigen (HLA), PSCA and MUC1. The passage numbers of each PDX for the patients were indicated. The negative controls (NC) are the liver tissues from a same mouse of third passage of PDX for patient P3. Scale bar = 20 μ m. PSCA and MUC1 detection results are also shown in Table 1.

Figure 3. Construction of anti-prostate stem cell antigen (PSCA) and anti-mucin 1 (MUC1) chimeric antigen receptor (CAR) T cells. (A) Structures of the genes used for lentiviral transfection. GFP, control without CAR; CAR-PSCA, anti-PSCA CAR; CAR-MUC1, anti-MUC1 CAR. (B) Representative flow cytometric analysis of

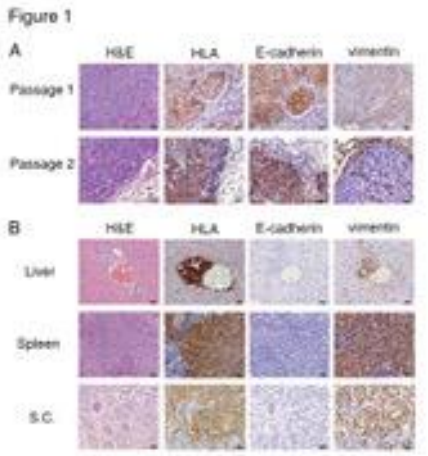
transfected T cells. (C) Reverse transcription-PCR detection of the following: β-ACTIN in wild type (WT), CAR-PSCA and CAR-MUC1 T cells (left); anti-PSCA scFv in WT and CAR-PSCA T cells and CAR-PSCA vector as a positive control (middle) and anti-MUC1 scFv in WT and CAR-MUC1 T cells and CAR-MUC1 vector as a positive control (right).

Figure 4. T cells expressing the prostate stem cell antigen (PSCA) or mucin 1 (MUC1) chimeric antigen receptor (CAR) specifically killed PSCA$^+$ or MUC1$^+$ lung cancer cell lines, respectively, in vitro. (A) Flow cytometric analysis of PSCA expression on A549, H23 and H460 cell lines. (B) Percentages of lung cancer line cells killed by GFP T cells and CAR-PSCA T cells at the indicated effector (E): target (T) ratios. The ratios were of the absolute number of CAR T cells vs target cells (corrected for transduction efficiency). T cells were cocultured with A549GL, H460GL, H23GL or H23-PSCA-GL cells for 18 h, and luciferase activities were measured using a D-luciferin substrate. % of killing = % (total activities without T cells – activities with T cells)/ total activities without T cells. Data were representative of killing assays using T cells from three different donors. (C) Results of enzyme-linked immunosorbent assays (ELISAs) to detect IL-2 and IFN-γ in the supernatants of cocultures at a E: T ratio of 1:1. (D) Flow cytometric analysis of MUC1 expression on A549, H23 and H460 cell lines. (E) Percentages of lung cancer line cells killed by GFP T cells and CAR-MUC1 T cells at the indicated E: T ratios. (F) Results of ELISAs to detect IL-2 and IFN-γ in the supernatants of cocultures at a E: T ratio of 1:1. Data were representative of killing assays using T cells from three

different donors. (G) Post-transfection GFP-Luciferase (GL) expression was detected in A549GL, H23GL and H460GL cell lines by flow cytometry. GFP served as a marker of luciferase expression. (H) Flow cytometric detection of PSCA (left) and MUC1 (right) in H23GL cells after lentiviral transduction. Error bars denote standard errors of the means, and groups were compared using the unpaired *t*-test. * P <0.05, ** P <0.01, *** P <0.001.

Figure 5. Prostate stem cell antigen chimeric antigen receptor (CAR-PSCA) expressing T cells inhibit the growth of non-small-cell lung cancer (NSCLC) and exhibit synergistic efficacy with mucin 1 CAR (CAR-MUC1) expressing T cells against NSCLC in patient-derived xenograft (PDX) models. (A) Diagram of the experiment with primary NSCLC tumors from patient P2 or P8 in NSI mice. Mice were inoculated subcutaneously with dissected tumor masses from patient P2 or P8 (2 mm × 2 mm), infused with 5×10^6 total T cells on days 7 and 10, and culled on day 40 for tumor analysis. B-E were results from the PDX model of patient P2. (B) T cells were analyzed for transfection efficiency before infusion into PDX mice of patient P2. (C) Tumor growth curves in groups treated with no T (n=3), GFP T (n=3), CAR-PSCA T (n=4) or CAR-MUC1 T (n=4) cells. (D) Tumors from mice treated with no T, GFP T, CAR-PSCA T or CAR-MUC1 T cells on day 40 are shown. One mouse from both the no T and GFP T groups died when tumors were small, which was not shown. (E) Comparison of the weights of tumors described in D. F-J were results from the PDX model of patient P8. (F) T cells were analyzed for transfection efficiency before infusion into PDX mice of patient P8. (G) Tumor growth curves in

groups treated with no T (n=3), GFP T (n=3), CAR-PSCA T (n=4), CAR-MUC1 T (n=4), and combinatorial CAR T cells (n=4). (H) Tumors from different groups in G on day 40 were shown. Also, both the no T and GFP T groups had one mouse died when tumors were small. (I) Comparison of the weights of tumors in H. (J) Tumors from CAR-PSCA T, CAR-MUC1 T and combinatorial groups were singled out for comparison. Error bars denote standard errors of the means, and groups were compared using the unpaired *t*-test. * P <0.05, ** P <0.01, *** P <0.001.



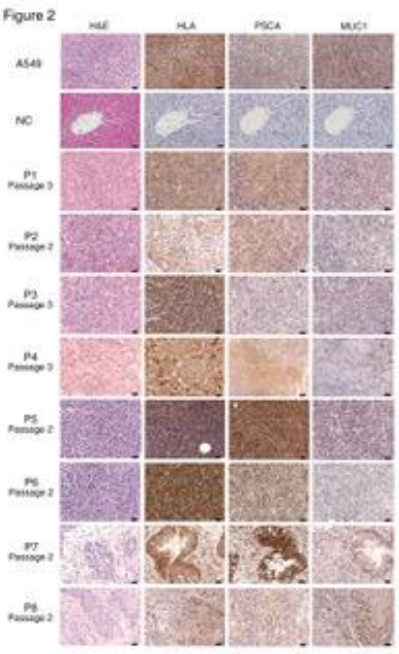


Figure 3

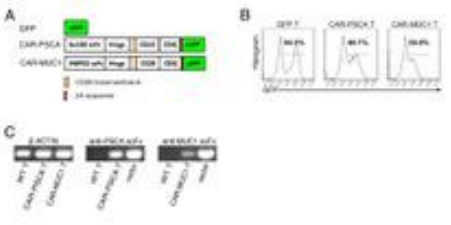


Figure 4

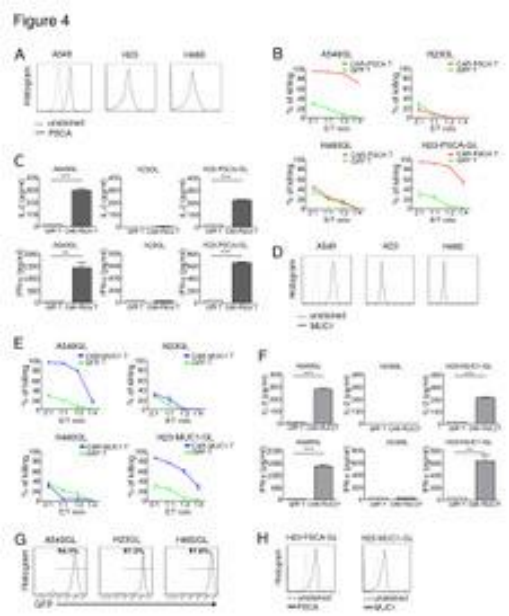


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Figure 5

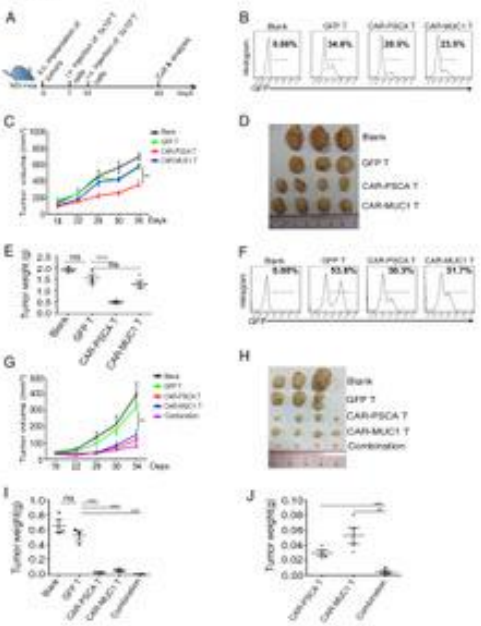

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Table 1

Clinical information of the patients and characteristics of the corresponding PDX models

Patient	Gender	Age	Pathology	PSCA	MUC1	E-cadherin (primary/PDX)	Vimentin (primary/PDX)	Metastasis in PDX	Route of administration
P1	M	60	AC	+	-	-/-	+/+	Yes	SC
P2	M	69	AC	+	-	+/+	-/-	No	IV
P3	M	65	AC	-	-	-/-	+/+	Yes	SC
P4	M	67	LCC	+	-	+/+	-/-	No	SC
P5	M	51	AC	+	-	ND	ND	No	SC
P6	F	57	AC	+	-	ND	ND	Yes	SC
P7	F	72	SCC	+	+	ND	ND	No	SC
P8	F	58	SCC	+	+	ND	ND	No	SC

M, male; F, female, AC, adenocarcinoma; LCC, large cell carcinoma; SCC, squamous cell carcinoma; +, expression; -, no expression; ND, not detected; SC, subcutaneous; IV, intravenous.